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<b>(54) Title:</b> ISOLATED PEPTIDES WHICH BIND TO HLA-B35 MOLECULES  <b>(57) Abstract</b>  The invention relates to peptides which bind to HLA-B35 molecules, leading to recognition and lysis of the resulting complexes by cytolytic T cells. Also a part of the invention are nucleic acid molecules which encode these peptides, and uses of each of these. The molecules are derived, in some cases, from tyrosinase, and portions of the tyrosinase molecule and portions of nucleic acid molecules which encode tyrosinase are also a part of the invention.		

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## ISOLATED PEPTIDES WHICH BIND TO HLA-B35 MOLECULES

### FIELD OF THE INVENTION

5 This invention relates to peptides which are presented by MHC molecules, leading to recognition by cytolytic T cells. More specifically, it relates to peptides which bind to HLA-B35 molecules, and are nonamers.

### BACKGROUND AND PRIOR ART

10 The process by which the mammalian immune system recognizes and reacts to foreign or alien materials is a complex one. An important facet of the system is the T cell response. This response requires that T cells recognize and interact with complexes of cell surface molecules, referred to as human leukocyte antigens ("HLA"), or major histocompatibility complexes ("MHCs"), and peptides. The peptides are derived from larger molecules which are processed by the cells which also present the HLA/MHC molecule. See in this regard Male et al., Advanced Immunology (J.P. Lipincott Company, 1987), especially chapters 6-10. The  
15 interaction of T cell and complexes of HLA/peptide is restricted, requiring a T cell specific for a particular combination of an HLA molecule and a peptide. If a specific T cell is not present, there is no T cell response even if its partner complex is present. Similarly, there is no response if the specific complex is absent, but the T cell is present. This mechanism is involved in the  
20 immune system's response to foreign materials, in autoimmune pathologies, and in responses to cellular abnormalities. Much work has focused on the mechanisms by which proteins are processed into the HLA binding peptides. See, in this regard, Barinaga, Science 257:880 (1992); Fremont et al., Science 257:919 (1992); Matsumura et al., Science 257:927 (1992); Latron et al., Science 257:964 (1992).

25 The mechanism by which T cells recognize cellular abnormalities has also been implicated in cancer. For example, in PCT application PCT/US92/04354, filed May 22, 1992, published on November 26, 1992, and incorporated by reference, a family of genes is disclosed, which are processed into peptides which, in turn, are expressed on cell surfaces, which can lead to lysis of the tumor cells by specific CTLs. The genes are said to code for "tumor rejection

antigen precursors" or "TRAP" molecules, and the peptides derived therefrom are referred to as "tumor rejection antigens" or "TRAs". See Traversari et al., Immunogenetics 35:145 (1992); van der Bruggen et al., Science 254:1643 (1991), both of which are incorporated by reference for further information on this family of genes.

5 In U.S. Patent No. 5,405,940, the disclosure of which is incorporated by reference, nonapeptides are taught which bind to the HLA-A1 molecule. The patent teaches that, given the known specificity of particular peptides for particular HLA molecules, one should expect a particular peptide to preferentially bind one particular HLA molecule, but not others. This is important, because different individuals possess different HLA phenotypes. As a result, while  
10 identification of a particular peptide as being a partner for a specific HLA molecule or class of HLA molecules has diagnostic and therapeutic ramifications, these are only relevant for individuals with that particular HLA phenotype. There is a need for further work in the area, because many cellular abnormalities are not restricted to one particular HLA phenotype, and targeted therapy requires some knowledge of the phenotype of the abnormal cells at issue.

15 The enzyme tyrosinase catalyzes the reaction converting tyrosine to dehydroxyphenylalanine or "DOPA" and appears to be expressed selectively in melanocytes (Muller et al., EMBO J 7:2715 (1988)). An early report of cDNA for the human enzyme is found in Kwon, U.S. Patent No. 4, 898,814. A later report by Bouchard et al., J. Exp. Med. 169:2029 (1989) presents a slightly different sequence. A great deal of effort has gone into  
20 identifying inhibitors for this enzyme, as it has been implicated in pigmentation diseases. Some examples of this literature include Jinbow, WO9116302; Mishima et al., U.S. Patent No. 5,077,059, and Nazzaropor, U.S. Patent No. 4,818,768. The artisan will be familiar with other references which teach similar materials.

25 Various U.S. Patent Applications incorporated by reference, teach that tyrosinase may be treated in a manner similar to a foreign antigen or a TRAP molecule - i.e., it was found that in certain cellular abnormalities, such as melanoma, tyrosinase is processed and a peptide derived therefrom forms a complex with HLA molecules on certain abnormal cells. These complexes were found to be recognized by cytolytic T cells ("CTLs"), which then lyse the presenting cells.

For example, allowed patent application Serial No. 08/583,238, filed January 5, 1996, teaches peptides which are derived from tyrosinase, and which complex to HLA-A2 and HLA-B44 molecules. Additional information on peptides derived from tyrosinase which are presented by HLA molecules may be found in U.S. Patent No. 5,487,974, and patent applications Serial No. 08/203,054, filed February 28, 1994, Serial No. 08/081,673, filed June 23, 1993 and Serial No. 07/994,928, filed December 22, 1992, and now abandoned. All of these references are incorporated by reference.

It is known that HLA-B35 molecules present peptides, with the resulting complexes being recognized by CTLs. See, in this regard, allowed U.S. patent application Serial No. 08/718, 964, filed September 26, 1996, and incorporated by reference. Other information on presentation by HLA-B35 molecules may be found in, e.g., Rammensee, et al., Immunogenetics 41:171 (1995), page 207 in particular, incorporated by reference. Also see Mason, et al., Tissue Antigens 51:417-465 (1998) incorporated by reference. Page 458 lists the amino acid sequences for the known HLA-B35 alleles, and shows that there is a great deal of identity there between.

New peptides have been identified which bind to HLA-B35 molecules, and are then recognized by CTLs. It is these peptides, and their use, which constitute the invention.

While derived from tyrosinase, the peptides of the invention need not be derived therefrom, as will be clear to the skilled artisan, and which will be seen from the disclosure which follows.

## **DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS**

### **EXAMPLE 1**

The melanoma cell line LG2-MEL, described by Degiovanni, et al., Eur. J. Immunol. 18:671-676 (1988), is recognized by autologous cytolytic T lymphocytes. At least three antigens are presented on its surface and are recognized by these CTLs; however, none of these antigens have been isolated or otherwise described. These experiments describe how the peptide recognized by "CTL 35-24" was identified.

First, in experiments not described herein, two monoclonal antibodies against HLA-B and HLA-C molecules were combined with CTL 35-24 and cell line LG2-MEL. These antibodies are described by Rebai, et al, Tissue Antigens 22:107-117 (1983), and Yang, et al., Immunogenetics 19:217-231 (1984) both of which are incorporated by reference. It was found that the antibodies inhibited lysis of LG2-MEL by CTL 35-24. Essentially, this was accomplished by adding dilutions of antibody (1/3-1/80) to the cytotoxicity assay. Since prior HLA typing had identified HLA-A24, A32, B35, B44, and Cw\*04 as the HLA molecules that the melanoma cell line presents, it was clear that the presenting molecule was either B35, B44, or Cw\*04.

It is well known that sublines of cancer cell lines can be derived, which present less than all of the HLA molecules of the parent line. One such subline of LG2-MEL, i.e. LG2-MEL 220, was known, which had lost expression of HLA-B35. CTL 35-24 failed to lyse this subline, suggesting that the presenting molecule is HLA-B35.

Subsequently, the cDNA for the HLA-B35 molecule was isolated, and sequenced, and found to be allelic subtype HLA-B\*3503. This subtype differs marginally from the other known HLA subtypes, as can be seen from Mason et al., supra. Hence, it is believed that the allelic subtypes are equivalent for purposes of peptide presentation

## EXAMPLE 2

As indicated in the "BACKGROUND" section, supra, melanoma cells are known to express a number of genes which are either not expressed or are expressed only in a restricted number of normal cells. These genes include MAGE genes, BAGE, GAGE (1-6), RAGE (1-4), LAGE, PRAME, tyrosinase, Melan-A, NY-ESO-1, pme/17, CASP-8, MUM-1, and gp100. Experiments were carried out to determine if the antigen presented by the HLA-B35 molecule was processed from one of these genes. To do this, cDNA for each of the above was obtained, following standard methods, and vectors prepared. The vectors were used to transfect COS cells, which were also transfected with cDNA for the HLA-B\*3503 molecule expressed by LG2-MEL. The cDNA used (i.e., cDNA for HLA-B\* 3503), was obtained from a cDNA library prepared from BB 49-SCCHN cells. This cell line is described by Mandruzzato, et al, J. Exp. Med. 186(5):785-793 (1997), incorporated by reference. The transfections were carried out using

50ng of each construct, described supra, using the well known DEAE/dextran method. Twenty four hours after transfection, CTL 35-24 (1500 cells), was added, and TNF production was measured, 24 hours later, using standard methods. See Traversari, et al, Immunogenetics 35:145-152(1992). Controls were used including cell line LG2-MEL 5-35 (positive control), and COS cells transfected with HLA-B\*3503 alone, or the melanoma associated gene alone. Only those cells which expressed both tyrosinase and HLA-B\*3503 stimulated TNF production.

### EXAMPLE 3

Once tyrosinase was identified as the processed molecule, studies were undertaken to determine the identity of the peptide presented by HLA-B\*3503. To do this, fragments of tyrosinase cDNA were prepared, following Wölfel, et al., Eur. J. Immunol. 24:759-764 (1994), and Brichard, et al., Eur. J. Immunol. 26:224-230 (1996), both of which are incorporated by reference, as well as U.S. Patent No. 5,487,974, also incorporated by reference. SEQ ID NO: 1 in this patent is SEQ ID NO: 40 of this application.

The same TNF assay as is described in example 2, supra, was used, except fragments of tyrosinase cDNA, rather than complete cDNA molecules, were used. Three fragments were positive, and these corresponded to nucleotides 1-1086, 427-1134, and 703-1134 of the coding region of tyrosinase cDNA. A fragment corresponding to position 574-831 was negative, leading to the conclusion that nucleotides 831-1086 encoded for the presented antigen. These correspond to amino acids 270-362 of tyrosinase, whose amino acid sequence is known. This amino acid sequence was compared to known peptides, which bind to HLA-B\*3501, and its binding motif, as described by Rammensee, et al., supra, incorporated by reference. This reference describes a binding motif for HLA-B35 which is a nonapeptide, where Pro is found at position 2, and Tyr is found at position 9. For decapeptides, Rammensee, et al, gives P2 and Y10 as anchors. HLA-B\*3501 was used because no information was found in the art for HLA-B\*3503. Rammensee et al. also gives Phe, Met, Leu, and Ile as auxiliary anchors for P9. The peptide defined by amino acid sequence LPSSADVEF (SEQ ID NO: 1) satisfies these requisites, and is found at amino acids 312-320 of tyrosinase. Its ability to stimulate lysis was tested by synthesizing the peptide, adding it to autologous lymphoblastoid cell line LG2-EBV which expresses HLA-B\*3503, and then adding CTL 35-24. Cell line HA7-EBV was also

tested. This line expresses HLA B\*3501. A  $^{51}\text{Cr}$  release assay was used, wherein cells were incubated with varying concentrations of the peptide of SEQ ID NO: 1. See U.S. Patent No. 5,519,117, incorporated by reference, for details of the assay. The  $^{51}\text{Cr}$  labeled cells were incubated for 30 minutes with the peptide, after which CTL 35-24 was added, in an effector (CTL) target (LG2-EBV) ratio of 5:1. The  $^{51}\text{Cr}$  release was measured after 3.5 hours.

The results are shown in figure 1. It is noted that the peptide provoked lysis of both types of cells, indicating that the peptide bound to both HLA-B\* 3501 and B\*3503. A dose of 1nM of peptide gave half maximal lysis of LG2-EBV cells and a dose of about 10nM of peptide gave half maximal lysis of HA7-EBV cells.

The foregoing examples describe the invention, which are peptides which bind to HLA-B35 molecules. These peptides are of formula

Leu Pro Xaa Xaa Xaa Xaa Xaa Xaa Phe

(SEQ ID NO: 2). Within this genus of peptides, those with position 3 as Ser, position 4 as Ser, position 5 as Ala, or position 6 as Asp, are preferred (SEQ ID NOS: 3-6). Peptides in accordance with the invention may have one or more of positions 3 - 6 as defined above. Positions 7 and 8 may be any amino acid. Also a part of the invention are peptides which correspond to the above referenced peptide, but are flanked at the N and C termini to no more than amino acids 270-312 and 321-362 of tyrosinase. In other words, peptides whose amino acids consist of no more than amino acids 270-311, concatenated to SEQ ID NO: 2, which is then concatenated to amino acids 321-362, respectively. Hence, peptides which consist of, e.g., amino acids 290-311 of tyrosinase, followed by SEQ ID NO: 2, followed by amino acids 321-340 of tyrosinase, are a part of the invention. Preferably, peptides no longer than about 16 amino acids and which comprise SEQ ID NO: 2 or SEQ ID NO: 1, or any of SEQ ID NOS: 4-6, are a part of the invention.

Also a facet of the invention are combinations of peptides which include at least the peptide of SEQ ID NO: 2, preferably one of the peptides of SEQ ID NOS: 1 and 3-6, together with one or more additional MHC or HLA binding peptides. It is known that individuals generally express six different HLA molecules on their cell surfaces. As the review of the art in the "BACKGROUND" section indicates, peptides which bind to other HLA molecules are known, as are other peptides which bind to HLA-B35 molecules. One can thus "customize"



compositions comprising two or more MHC binding peptides, wherein at least one of these binding peptides is a peptide defined by SEQ ID NOS: 1-6.

Also a part of the invention are nucleic acid molecules which encode the peptides of the invention, such as a nucleic acid molecule consisting of nucleotides which encode no more than amino acids 270-362 of tyrosinase, and no less than a peptide as defined by SEQ ID NO: 2, or more preferably, one of SEQ ID NOS: 1 and 3-6. These nucleic acid molecules can be incorporated into expression vectors, and the nucleic acid molecules or vectors can be used to transform or transfect cells, cell lines, and cell strains, be these eukaryotic or prokaryotic. They can also be used in combination with nucleic acid molecules which encode an MHC molecule, such as an HLA-B35 molecule, such as HLA B\*3501 or HLA-B\*3503.

The peptides and nucleic acid molecules of the invention have various uses, which are also a part of the invention. For example, in addition to their usefulness in therapeutic applications, such as the generation of cytolytic T cells, either in vitro or in vivo, which specifically lyse pathogenic cells, the peptides can be used to identify HLA-B35 positive cells, or to remove HLA-B35 positive cells from mixtures containing such cells. The nucleic acid molecules can be used, inter alia, as probes to identify cells which are expressing tyrosinase.

Also a part of the invention is a multicomponent complex useful, e.g., in isolating cytolytic T cells specific for a particular target, from a sample. The complex comprises a first binding partner and a second binding partner, wherein the first and second binding partner are specific for each other. These can be, e.g., avidin or streptavidin and biotin, an antibody or a binding portion of an antibody specific to biotin, and so forth. The key feature is that the second binding partner must be bound to a plurality of complexes of an MHC molecule, a  $\beta 2$  microglobulin molecule and a peptide which binds specifically to said MHC molecule, and the multicomponent complex must be labeled. The MHC molecules are preferably HLA molecules, such as HLA-B35 molecules, but, it will be understood by the artisan of ordinary skill that any HLA molecule could be used. With respect to the peptides of interest, many references, including review articles, U.S. and non-U.S. patents, and so forth describe peptides beyond SEQ ID NOS: 2-6 and their binding partner HLA molecule. All are encompassed by the invention. Exemplary peptides and their HLA molecule partners are presented later in this application.

Preferably, the second binding partner is biotin, but it may also be, e.g., an antibody which is specific for a component of the HLA/β2 microglobulin/peptide complex, such as an HLA specific antibody, or a β2 microglobulin specific antibody. Similarly, the first binding partner may be e.g., recombinant or naturally occurring protein L, recombinant or naturally occurring protein A, or even a second antibody. The complex can be in soluble form, or bound, e.g., to a removable solid phase, such as a magnetic bead.

The number of HLA/β2 microglobulin/peptide complexes in the large molecule of the invention may vary. It comprises at least two complexes, and preferably at least four, but more may be present as well.

The complex of binding partners and HLA/β2 microglobulin/peptide may be labeled, using any of the labels known to the art. Examples of fluorescent labels are given supra. Enzymatic labels, such as alkaline phosphatase, metal particles, colored plastics made of synthetic materials, radioactive labels, etc., may all be used.

A third binding partner may also be used, which binds, specifically, to the first binding partner. For example, if the first binding partner is streptavidin, and the second binding partner is biotin, then the third binding partner may be a streptavidin specific antibody. When three or more binding partners are used, the label referred to supra may be attached to any of the binding partners so long as engagement with the HLA/β2 microglobulin/peptide complexes is not impaired.

The complexes may be used, e.g., to identify or to isolate cytolytic T cells present in a sample, where these cells are specific for the HLA/β2 microglobulin/peptide complex. As the examples show, such cytolytic T cells bind to the immunocomplexes of the invention. In a preferred embodiment, the sample being tested is treated with a reactant which specifically binds to a cytolytic T cell, wherein said label provides a detectable signal. The sample, including labeled CTLs, is then contacted to the complex, where it binds, and can be separated via any of the standard, well known approaches to cell separation. Preferably, FACS is used, but other separation methodologies will be known to the skilled artisan as well. The peptide used is left to the needs of the skilled artisan, and will depend, e.g., on the nature of the specific MHC system under consideration, a table of exemplary, but no means the only, peptides for which CTLs are known, follows. These are also set forth in SEQ ID NOS: 7-38.

Gene	MHC	Peptide	SEQ ID
MAGE-1	HLA-A1	EADPTGHSY	7
	HLA-Cw16	SAYGEPRKL	8
MAGE-3	HLA-A1	EVDPIGHLV	9
	HLA-A2	FLWGRPALV	10
	HLA-B44	MEVDPIGHLV	11
BAGE	HLA-Cw16	AARAVFLAL	12
GAGE-1,2	HLA-Cw16	YRPRPRRY	13
RAGE	HLA-B7	SPSSNRIRNT	14
GntV	HLA-A2	VLPDVFIRC(V)	15
MUM-1	HLA-B44	EEKLIVVLF	16
		EEKLSVVLF	17
CDK4	HLA-A2	ACDPHSGHFV	18
		ARDPHSGHFV	19
$\beta$ -catenin	HLA-A24	SYLDSGIHF	20
		SYLDSGIHF	21
Tyrosinase	HLA-A2	MLLAVLYCL	22
	HLA-A2	YMNGTMSQV	23
	HLA-A2	YMNGTMSQV	24
	HLA-A24	AFLPWHRLF	25
	HLA-B44	SEIWRDIDF	26
	HLA-B44	YEIWRDIDG	27
	HLA-DR4	QNILLSNAPLGPGFP	28
	HLA-DR4	DYSYLQDSDPDSFQD	29
Melan-A <sup>Mart-1</sup>	HLA-A2	(E)AAGIGILTV	30
	HLA-A2	ILTVILGVL	31
gp100 <sup>Pmel117</sup>	HLA-A2	KTWGQYWQV	32
	HLA-A2	ITDQVPFSV	33

	HLA-A2	YLEPGPVTA	34
	HLA-A2	LLDGTATLRL	35
	HLA-A2	VLYRYGSFSV	36
DAGE	HLA-A24	LYVDSLFFL	37
MAGE-6	HLA-Cw16	KISGGPRISYPL	38

Additional peptides may be found, e.g., in U.S. patent application Serial Nos. 08/672,351, 08/669,590, 08/487,135, now U.S. Patent No. 08/530,569, and 08/880,693, and 08/718,964, now U.S. Patent No. \_\_\_\_\_, all of which are incorporated by reference.

A further aspect of the invention are so-called "mini genes" which carry information necessary to direct synthesis of peptides via cells into which the mini genes are transfected. Mini genes can be designed which encode one or more antigenic peptides, and are then transferred to host cell genomes via transfection with plasmids, or via cloning into vaccinia or adenoviruses. See, e.g., Zajac, et al., *Int. J. Cancer* 71:496 (1997), incorporated by reference.

The peptides of the invention may be combined with peptides from other tumor rejection antigens to form 'polytopes'. Exemplary peptides include those listed in the applications set forth supra.

Additional peptides which can be used are those described in the following references, all of which are incorporated by reference: U.S. Patent Nos. 5,405,940; 5,487,974; 5,519,117; 5,530,096; 5,554,506; 5,554,724; 5,558,995; 5,585,461; 5,589,334; 5,648,226; and 5,683,886; PCT International Publication Nos. 92/20356; 94/14459; 96/10577; 96/21673; 97/10837; 97/26535; and 97/31017 as well as pending U.S. Application Serial No. 08/713,354. These peptides may also be combined with peptides that complex with MHC-Class II molecules, such as peptides derived from tumor rejection antigen precursors as is described in Serial No. 08/927,015, and a continuation in part application to Knuth et al., filed on October 2, 1998, as a CIP of Serial No. 09/062,422. This newly filed CIP is incorporated by reference.

Polytopes are groups of two or more potentially immunogenic or immune stimulating peptides, which can be joined together in various ways, to determine if this type of molecule will stimulate and/or provoke an immune response.

These peptides can be joined together directly, or via the use of flanking sequences. See Thompson et al. Proc. Natl. Acad. Sci. USA 92(13):5845-5849 (1995), teaching the direct linkage of relevant epitopic sequences. The use of polytopes as vaccines is well known. See, e.g., Gilbert et al., Nat. Biotechnol. 15(12):1280-1284 (1997); Thompson et al., supra; Thompson et al., J. Immunol. 157(2):822-826 (1996); Tam et al., J. Exp. Med. 171(1):299-306 (1990), all of which are incorporated by reference. The Tam reference in particular shows that polytopes, when used in a mouse model, are useful in generating both antibody and protective immunity. Further, the reference shows that the polytopes, when digested, yield peptides which can be and are presented by MHCs. Tam shows this by showing recognition of individual epitopes processed from polytope 'strings' via CTLs. This approach can be used, e.g., in determining how many epitopes can be joined in a polytope and still provoke recognition and also to determine the efficacy of different combinations of epitopes. Different combinations may be 'tailor-made' for the patients expressing particular subsets of tumor rejection antigens. These polytopes can be introduced as polypeptide structures, or via the use of nucleic acid delivery systems. To elaborate, the art has many different ways available to introduce DNA encoding an individual epitope, or a polytope such as is discussed supra. See, e.g., Allsopp et al., Eur. J. Immunol. 26(8):1951-1959 (1996), incorporated by reference. Adenovirus, pox-virus, Ty-virus like particles, plasmids, bacteria, etc., can be used. One can test these systems in mouse models to determine which system seems most appropriate for a given, parallel situation in humans. They can also be tested in human clinical trials.

Also a feature of the invention are compositions which comprise at least one of the peptides of the invention, in combination with at least one adjuvant. Such compositions can be used, e.g., to generate immune responses, preferably in humans, as part of a therapeutic regime, but also in subject non-human animals, to generate immune components which can then be used to treat humans, or diagnostically. The artisan of ordinary skill is familiar with such adjuvants, and thus these do not have to be set forth here.

These compositions can also include so-called co-stimulatory molecules. These are molecules which are proteins, or encode proteins, that interact with molecules on the surface of T cells, thereby co-stimulating a T cell already stimulated by formation of an MHC molecule/antigen/T cell receptor interaction. Such co-stimulatory molecules enhance antitumor

immunity, and CTL proliferation. Exemplary of such co-stimulatory molecules are those known as "B7-1" and "B7-12," or CD80 and CD86, respectively. See Zhang, et al, Proc. Natl. Acad. Sci. USA 95(11):6284-6289 (1998), incorporated by reference.

Such co-stimulatory molecules can be combined with, e.g. interleukins, such as IL-6 and IL-12. See Gajewski, et al, J. Immunol 154:5637-5648 (1995). As noted, supra, the co-stimulatory molecules may be administered in the form of a nucleic acid molecule. Such an approach can be useful in connection with CTL expansion for adoptive transfer immunotherapy (Wang et al, J. Immunother. Emphasis Tumor Immunol. 19:1-8 (1996)). The requisite nucleic acid molecules can be administered in the form of "naked" DNA (Kim et al, Nat. Biotechnol 15(7):641-646 (1997)), as well as in the form of recombinant vectors, such as adenovirus and pox virus vectors. See Wendtner et al, Gene Ther. 4(7):726-735 (1997). All of these systems can be adapted so that the co-stimulatory molecule is expressed together with other molecules of choice, including the peptides, adjuvant molecules, and so forth.

In addition to the foregoing, antibodies can function as co-stimulatory molecules, as these can act as ligands to cell receptors, thereby costimulating the cell. The B7 molecules discussed supra are ligands for CD28 molecules. Hence, anti CD28 antibodies, be these polyclonal, monoclonal, humanized, etc., can all act in this fashion.

In addition to B7 molecules, lymphocyte function associated antigen-1 (LFA-1), CD40L and anti-CD40 antibodies can also be used as co-stimulatory molecules. These are all exemplary of the family of co-stimulatory molecules, and should not be regarded as the only possible alternatives.

Also a feature of the invention is the use of these peptides to determine the presence of cytolytic T cells in a sample. It was shown, supra, that CTLs in a sample will react with peptide/MHC complexes. Hence, if one knows that CTLs are in a sample, HLA-B35 positive cells can be "lysed" by adding the peptides of the invention to HLA-B35 positive cells, such as HLA-B\*3503 positive cells, and then determining, e.g., radioactive chromium release, TNF production, etc. or any other of the methods by which T cell activity is determined. Similarly, one can determine whether or not specific tumor infiltrating lymphocytes ("TILs") are present in a sample, by adding one of the claimed peptides with HLA-B35 positive cells to a sample, and determining lysis of the HLA-B35 positive cells via, e.g., <sup>51</sup>Cr release, TNF presence and so

forth. In addition, CTL may be detected by ELI-SPOT analysis. See for example Schmittle et al. (1997). J. Immunol. Methods 210:167-174 and Lalvani et al. J. Exp. Med. 126:859 (1997) or by FACS analysis of fluorogenic tetramer complexes of MHC Class I/peptide (Dunbar et al. (1998), Current Biology 8:413-416. All are incorporated by reference.

5           Of course, the peptides may also be used to provoke production of CTLs. As was shown, supra, CTL precursors develop into CTLs when confronted with appropriate complexes. By causing such a "confrontation" as it were, one may generate CTLs. This is useful in an in vivo context, as well as ex vivo, for generating such CTLs.

10           Other features of the invention will be clear to the skilled artisan, and need not be repeated here.

          The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

**CLAIMS**

1. An isolated peptide which consists of amino acid sequence  
Leu Pro Xaa Xaa Xaa Xaa Xaa Xaa Phe  
(SEQ ID NO: 2), wherein each Xaa is any amino acid.
2. The isolated peptide of claim 1, wherein at least one of the following criteria are satisfied:  
the first Xaa is Ser (SEQ ID NO: 3)  
the second Xaa is Ser (SEQ ID NO: 4)  
the third Xaa is Ala (SEQ ID NO: 5)  
the fourth Xaa is Asp (SEQ ID NO: 6)
3. The isolated peptide of claim 1, wherein the peptide consists of the amino acid sequence  
of SEQ ID NO: 1.
4. The isolated peptide of claim 2, wherein the fifth amino acid is Val, or the sixth amino  
acid is Glu.
5. An isolated peptide the amino acid sequence of which consists of at least the amino acid  
sequence set forth in SEQ ID NO: 2, and no more than amino acids 270-311 of tyrosinase  
concatenated to SEQ ID NO:2, concatenated to no more than amino acids 321-362 of tyrosinase.
6. The isolated peptide of claim 5, consisting of amino acids 270-362 of tyrosinase.
7. Composition comprising the isolated peptide of claim 1, and an adjuvant.
8. Composition comprising the isolated peptide of claim 2, and an adjuvant.
9. Composition comprising the isolated peptide of claim 3, and an adjuvant.



10. Composition comprising the isolated peptide of claim 4, and an adjuvant.
11. Composition comprising the isolated peptide of claim 5, and an adjuvant.
12. Composition comprising the isolated peptide of claim 6, and an adjuvant.
13. Composition comprising the isolated peptide of claim 1, and a peptide which binds to an MHC Class II molecule.
14. An isolated nucleic acid molecule which encodes the isolated peptide of claim 1.
15. An isolated nucleic acid molecule which encodes the isolated peptide of claim 3.
16. An isolated nucleic acid molecule which encodes the isolated peptide of claim 5.
17. An isolated nucleic acid molecule which encodes the isolated peptide of claim 6.
18. Expression vector comprising the isolated nucleic acid molecule of claim 14, operably linked to a promoter.
19. Expression vector comprising the isolated nucleic acid molecule of claim 15, operably linked to a promoter.
20. Expression vector comprising the isolated nucleic acid molecule of claim 16, operably linked to a promoter.
21. Expression vector comprising the isolated nucleic acid molecule of claim 17, operably linked to a promoter.

22. Recombinant cell, cell line, or cell strain, comprising the isolated nucleic acid molecule of claim 14, 15, 16, or 17.
23. Recombinant cell, cell line, or cell strain, comprising the expression vector of claim 18, 19, 20, or 21.
24. The recombinant cell, cell line, or cell strain of claim 22, further comprising a nucleic acid molecule which encodes an HLA-B35 molecule.
25. The recombinant cell, cell line or cell strain of claim 23, further comprising a nucleic acid molecule which encodes an HLA-B35 molecule.
26. Composition comprising the peptide of claim 1, and at least another MHC binding peptide.
27. Kit useful in generating cytolytic T cells, comprising a separable portion of each of the isolated nucleic acid molecule of claim 14 and an isolated nucleic acid molecule which encodes an HLA-B35 molecule.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/23038

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/197.11, 198.1; 435/ 252.3, 325, 455, 471; 530/328, 324; 514/12, 15; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DERWENT, EMBASE, MEDLINE, SCISEARCH, CANCERLIT, CHEM ABS, tyrosinase, HLA-B35, CTL, binding motif

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCHONBACH et al. Refined Peptide HLA-B*3501 Binding Motif Reveals Differences In 9-mer To 11-mer Peptide Binding. Immunogenetics. 1996, Vol. 45, pages 121-129, see entire article.	1-27
Y	US 4,898,814 A (KWON et al.) 06 February 1990, see entire document.	1-27
Y	US 5,519,117 A (WOLFEL et al.) 21 May 1996, see entire document.	1-27
Y	US 5,487,974 A (BOON-FALLEUR et al.) 30 January 1996, see entire document.	1-27
Y	5,756,666 A (TAKIGUCHI et al.) 26 May 1998, see entire document.	1-27



Further documents are listed in the continuation of Box C.



See patent family annex.

* *A* *B* *L* *O* *P*	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier document published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	*T* *X* *Y* *A*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art document member of the same patent family
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Date of the actual completion of the international search

04 JANUARY 2000

Date of mailing of the international search report

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Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

MARSHA LUBET

Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/23038

A. CLASSIFICATION OF SUBJECT MATTER:  
IPC (6):

A61K 38/08, 39/00, 39/385; C12N 1/21, 5/10, 15/63, 15/70, 15/85, 15/87, 15/74; C07K 19/00

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/197.11, 198.1; 435/ 252.3, 325, 455, 471; 530/328, 324; 514/12, 15; 536/23.5